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Flow Cytometry APC-Tandem Dyes Are Degraded Through a Cell-Dependent Mechanism

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Abstract

Technological developments of multiparametric flow cytometry come along with the generation of new dyes. The APC-tandem dyes, which combine the fluorophores APC and Cy7/H7, allow the detection of a specific signal in the APC-Cy7/H7 channel along with an unexpected nonspecific signal in the APC channel. Depending on the magnitude of the latter, it may be a handicap for interpreting the data of multicolor labeling experiments. We investigated the alteration of the APC-tandem dyes by labeling peripheral blood cells with antibodies directed toward leukocyte surface proteins and by analyzing cells by flow cytometry. Our results show that the APC-Cy7/H7 tandem fluorochromes degraded over time. Nonspecific APC signal was observed with the various antibodies tested only upon cell attachment but not under bead linkage. Moreover, the percentage of degradation of the APC-Cy7/H7 dyes was dependent on the cell type analyzed. Interestingly, nonspecific APC signal strongly decreased when the metabolic activity of immunolabeled cells was inhibited or when cells were incubated with vitamin C. This study demonstrates that the APC-tandem dyes are the target of cell-dependent degradation, which may be antagonized. These findings will allow cytometer users to optimize their multicolor panels. © 2009 International Society for Advancement of Cytometry

Key terms

flow cytometry; leukocytes; cell surface marker antibodies; APC-tandem fluorochromes; compensation; degradation; fixation; temperature; sodium azide; hydrogen peroxide; vitamin C

THE flow cytometry methodology allows simultaneous measurement of distinct fluorescent and morphological parameters at the cellular level. Development of multicolor systems (1–4) has numerous advantages such as providing a large amount of information at the single-cell level, an accurate measurement even in rare cell populations, and identification of new cellular characteristics. Application of multicolor panels is particularly rewarding in clinical settings as it is used in routine diagnosis and prognosis evaluation in numerous diseases (5–7). Indeed, this technology is crucial for the functional identification of cell subsets within the whole hematopoietic system and provides a better understanding of both cell physiology and pathophysiology of hematological malignancies (8,9).

Exploration of multiple biomarkers by flow cytometry at the single-cell level results in increased demand for new dyes, which may be used simultaneously (10–14). Performance of multiparametric analysis has been significantly enhanced with the introduction of the APC-based tandem dyes. The latter can be coupled to antibodies and emit in red and far-red regions of light spectrum. APC-Cy7 (allophycocyanin-cyanine 7) and its analog APC-H7 (allophycocyanin-Hilite®7-BD) (11,12) are APC-tandem dyes that exhibit similar spectral properties with maximum absorption at ~650 nm. When receiving excitation from a red laser (HeNe, 633 nm), the APC fluorochrome transfers energy to the cyanine dye that in turn presents a peak of emission at 767 nm (15). However, excitation of these donor–acceptor dye

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combinations might present disadvantages, including unquenched donor fluorescent signal, the nonenhanced acceptor intensity, and the efficiency level of energy transfer.

In parallel to the development of these dyes, many standards have been engineered for the adequate set up of the machine and the appropriate interpretation of the results (16). Among these standards, CompBeads® are considered to mimic cells by binding to fluorochrome-conjugated antibodies. Therefore, microbeads allow measurement of fluorochrome emission spectra in the same environmental media as labeled cells (3,16,17).

In this study, using single or multicolor labeling flow cytometry, we analyzed the APC signal coming from APC-Cy7 and -H7 tandem conjugates antibodies on microbeads and human leukocytes over time. Presence of such APC signal is referred to as "decoupling phenomenon" in this manuscript. By using different reagents and protocols, we tested and improved the stability of such tandem dyes. On the basis of our data, we provide flow cytometry users with guidelines for design and optimization of their multicolor panels containing APC-tandem dyes.

Materials and Methods

Materials and Cells

Antibodies anti-CD45, -CD20, -CD3, -CD19 conjugated to APC-Cy7 and -H7, CD14 conjugated to FITC; -CD20 to PE, -CD5 to PerCPCy5.5, -CD33 to PE-Cy7, -CD19 to APC, as well as BD Phosflow Lyse/Fix buffer 5× and Compbeads® were purchased from BD Biosciences; Lysis buffer pH 7.2 [155 mM NH_4CL (Sigma), 10 mM $KHCO_3$ (Sigma), and 0.1 mM EDTA (Prolabo)]; phosphate buffered saline (PBS) from Biomérieux; fetal bovine serum (FBS), heated for 30 min at 56°C, from Pan Biotech Gmbh; L-ascorbic acid (vitamin C), sodium azide (NaN_3), and hydrogen peroxide (H_2O_2) from Sigma-Aldrich.

Peripheral blood samples tested were drawn from control subjects selected on the basis of a normal blood cell count and absence of treatment for hematological disease, ages ranged from 21 to 100 and sex-ratio was close to 1. One blood sample has been tested in each experiment presented in this manuscript, and three to four blood samples have been used for the various repeats.

PRI, GRANTA, and JeKo cell lines were cultured at 37°C in a humidified 5% CO^2 atmosphere. PRI and GRANTA cell lines were generously provided by Pr. F. Praz (Cellular and Molecular Oncology, Bobigny, France) and Dr. B. Sola (Molecular and Cellular biology of the signal, Caen, France), respectively. Conditions of PRI cell line culture were previously described by Baran-Marszak et al. (18). The GRANTA cell line (human B cell line derived from a leukemic transformation of mantle cell lymphoma) was maintained in 90% Dulbecco's MEM (4.5 g/l glucose) + 10% FBS + 2 mM L-glutamine (Sigma-Aldrich). JeKo cell line was established from peripheral blood mononuclear cells of a patient with a large cell variant of mantle cell lymphoma. This B-cell line (negative for Epstein-Barr virus and express IgM) was purchased from ATCC and was grown in RPMI-1640 medium with 20% FBS.

Methods

Aliquots of human peripheral blood (containing 500,000 leukocytes) were incubated for 10 min in lysis buffer pH 7.2 and washed three times with PBS 1× supplemented with 2.5% FBS (PBS/FBS). In single-color labeling, cells were then immunolabeled with 5 μl of the indicated antibodies in 100 μl incubation buffer for 20 min at room temperature, washed three times, and resuspended in 200 μl PBS/FBS containing appropriate products (fixative solution, NaN_3, H_2O_2, or vitamin C). Dead cells were excluded from the flow cytometry analysis using the double-strand nucleic acid intercalant 7-aminoactinomycin-D (7-AAD) (Sigma-Aldrich), according to the manufacturer's instruction. In multiparametric flow cytometry, cells were labeled with a mixture of antibodies (10 μl of anti-CD14 conjugated to FITC; -CD20 to PE, -CD5 to PerCPCy5.5, and 2.5 μl of anti-CD33 conjugated to PE-Cy7, -CD19 to APC, and -CD3 to APC-Cy7). For incubation experiments, before each time point, cells were mixed and subjected to flow cytometry analysis (FACSCanto™ II Becton Dickinson). Compbeads® is a reagent used for automated calculation of compensation matrix in multiparametric flow cytometry experiments. As an alternative, the matrix may be determined manually by setting the compensation for each fluorochrome by aligning the median fluorescence intensity of labeled and unlabeled beads for each fluorescence channel. From 10,000 to 50,000 events were acquired per test. Singlet cells were selected by commonly used cell sorting procedure: intersection of the three gates labeled as FCS-A vs. SSC-A, FSC-H vs. FSC-W, and SSC-H vs. SSC-W dot plots, respectively. For each cell type, percentage of decoupling was determined by calculating the ratio between the number of APC-positive cells and the number of APC-tandem-positive cells (APC^+/APC-Cy7/H7 $tandem^+$ × 100) using the BD FACSDIVA™ software (BD Biosciences). The figures presented in this manuscript show a representative experiment of three to four repeated experiments.

Results

APC-Cy7 and APC-H7 Tandem Dyes Conjugated to Anti-CD45 Antibody Degrade Over Time

Use of APC-tandem conjugates can lead to uncontrolled emission in the APC channel. To characterize this parasite signal, we used conjugated antibodies directed toward leukocyte surface proteins.

First, we labeled peripheral blood cells with an anti-CD45 antibody conjugated to APC-Cy7 or H7 fluorochromes and analyzed by flow cytometry the APC-Cy7/H7-positive cells over time (Fig. 1 and Supporting Information Fig. S1). Panleukocyte anti-CD45 antibodies were coupled to APC-tandem dyes labeled lymphocytes, monocytes, and polymorphonuclear leukocytes (19) (Figs. 1A and 1B, left panels). By targeting lymphocytes and generating two-dimensional dot plots based on APC-Cy7/H7 fluorescence intensity vs. APC

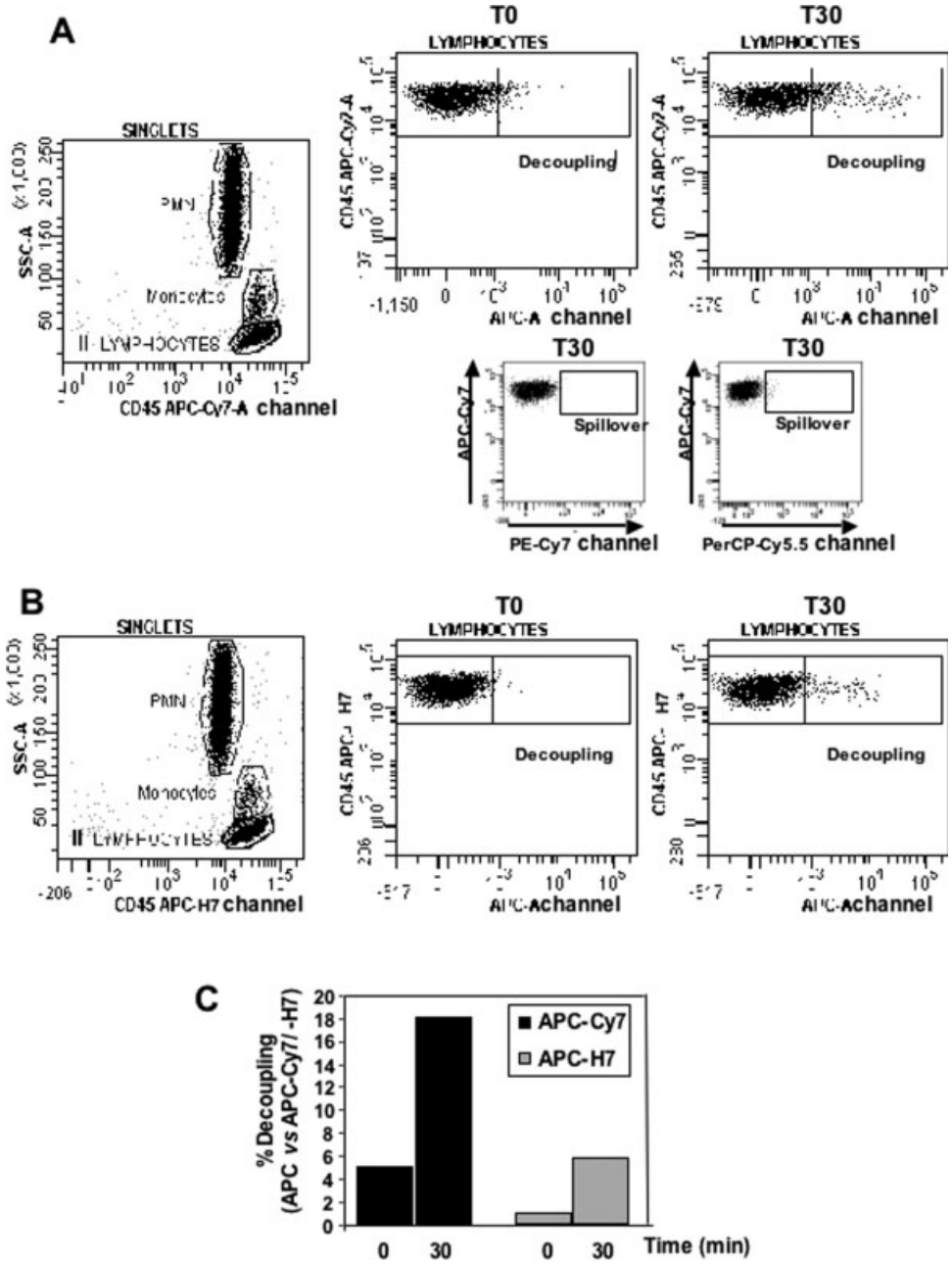

Figure 1. APC-Cy7 and APC-H7 tandem dyes conjugated to an anti-CD45 antibody degrade over time. Blood cells were immunolabeled with anti-CD45 antibody conjugated to APC-Cy7 (**A**) or APC-H7 (**B**). The dot plots presented show compensated data. Lymphocytes, monocytes, and polymorphonuclear (PMN) leukocytes are targeted on the dot plots CD45 APC-Cy7/H7 vs. SSC (Left panels; A and B) based on CD45 expression intensity. The dot plots APC vs. CD45-APC-Cy7 (A) or -APC-H7 (B) show CD45 APC-Cy7/H7-positive lymphocytes in APC channel. (**C**) Percentage of decoupling (APC vs. APC-Cy7 (A) or APC-H7 (B)) at time 0 (T0) and 30 min (T30) is determined by calculating the ratio between the number of APC-positive cells (indicated as ''decoupling'') and total number of APC-tandem dye-positive cells and graphed.

fluorescence intensity, our compensated data showed that at initial time (T0), the lymphocytes appeared as an uniform cell population, which was highly positive in the APC-Cy7/H7 channel (Figs. 1A and 1B, middle panels). To evaluate this alteration, we targeted the APC-positive subpopulation within the APC-Cy7/H7-positive lymphocyte population (indicated as "decoupling" in Fig. 1). Positivity threshold was determined in the APC channel at T0 and reported at T30. Percentage of "decoupling" cells was expressed as the ratio of total numbers of APC/APC-tandem double positive over total APC-tandem-positive lymphocytes for both time points. As shown in Figure 1C, the percentage of decoupling of the

APC-Cy7 and APC-H7 fluorochromes was increased at T30 when compared with T0. Of note, similar results were obtained with an anti-CD45 antibody coupled to APC-Alexa 750 (data not shown). We observed that basal percentage of decoupling (T0) was higher for the APC-Cy7 tandem dye than for APC-H7 tandem fluorochrome, but ratios of decoupling of both APC-tandem dyes had a similar variation during incubation time (four- to fivefold; Fig. 1C).

To better characterize this uncontrolled emission in the APC channel, we performed a series of controls supplied in Supporting Information Figure S2. When compensation settings were not applied, we observed a distortion of APC-Cy7/H7 cell population in the APC, PE-Cy7, and PerCP-Cy5.5 dotplots (Supporting Information Fig. S2, first line). Once compensation values were determined by aligning the median of fluorescent intensities (MFI) of the APC-Cy7/H7 antibody-bound (dark gray) and unbound beads (light gray) in the indicated channels (Supporting Information Fig. S2, second and third lines), we corrected the spillover from APC-Cy7/H7-positive cells into APC, PE-Cy7, and PerCP-Cy5.5 channels (Supporting Information Fig. S2, forth line). However, even after compensation of raw data APC-Cy7 or H7-positive cell populations presented a weak APC signal at initial time (Figs. 1A and 1B; T0 panels). Interestingly, upon incubation of 30 min at room temperature and in the dark, our compensated data (settings adjusted on beads at time 30 min) revealed that the APC-Cy7/H7-positive cell population extended into the APC channel with either little or no spreading in the PE-Cy7 and PerCP-Cy5 channels (Figs. 1A and 1B; T30 panels, Supporting Information Fig. S1). These results suggested that the increased signal detected in APC channel over time was related to an alteration of the APC-tandem dyes rather than emanating from a spillover of the APC-tandem fluorochromes.

Altogether, these data demonstrate that the APC-tandem dyes conjugated to anti-CD45 antibodies were subjected to degradation over time in lymphocytes, monocytes, and polymorphonuclears.

APC-Cy7 and APC-H7 Tandem Fluorophore Degradation Is Dependent on Cell Type

Next, we further delineated the influence of the cellular context on the decoupling phenomenon by analyzing the relative stabilities of the APC-tandem dyes on lymphocytes compared with Compbeads® (referred to as beads in the text). Lymphocytes and beads were separately labeled with anti-CD45 antibodies coupled to APC-Cy7 or APC-H7 tandem fluorophores. As illustrated in Figure 2A (left panel), antibodies recognized the cellular markers through their antigenic binding site, whereas binding to the microparticles (Compbeads®) was via light chains recognition. Interestingly, percentage of decoupling of APC-Cy7 and APC-H7 tandem dyes was increased over time for cell-bound antibodies only. No obvious decoupling was observed for bead-coupled antibodies (Fig. 2A middle and right panels). Similar results were observed with other antibodies, such as anti-CD20 antibody (data not shown). These data suggest that the alteration of the APC-tandem dyes was related to the presence of cells rather than an intrinsic instability in our experimental conditions.

To test this hypothesis, beads were labeled with anti-CD45 antibodies coupled to APC-Cy7 and incubated in the absence or presence of unlabeled cells. According to our previous results, beads alone do not show apparent decoupling (Figs. 2A and 2B, beads alone). In contrast, when labeled beads were incubated with unlabeled cells, APC-tandem dye degradation occurred on antibody-bound beads (Fig. 2B, beads plus cells). This strongly suggests that the APC-tandem fluorochrome alteration results from the presence of cells.

To verify whether the APC-tandem dye degradation occurred regardless of the antibody used, several antibodies directed toward lymphocyte membrane markers and conjugated to either APC-Cy7 or -H7 tandem fluorochromes were tested. Both APC-Cy7 and -H7 tandem fluorochromes conjugated to antibodies toward B cell (anti-CD20 or anti-CD19) or T cell (anti-CD3) markers showed degradation over incubation time (Supporting Information Fig. S3 and data not shown). Similar to our previous data (Fig. 1), levels of degradation of the APC-Cy7 tandem were higher than those of APC-H7 tandem fluorophores. These results also confirmed that alteration of APC-tandem dyes occurred regardless of targeting B or T lymphocyte surface markers.

Influence of cell type was then further delineated. Therefore, leukocyte populations were labeled with the Pan-leukocyte anti-CD45 antibodies conjugated to either APC-Cy7 or APC-H7 fluorochromes, and the decoupling in each cell subpopulation was determined over time. As presented in Figure 2C, the decoupling percentage of the APC-Cy7 and APC-H7 tandem dyes was increased when anti-CD45 antibodies were bound to lymphocytes, monocytes, or granulocytes. However, for both APC-tandem conjugates, the rate of degradation was gradually increased among lymphocytes, granulocytes, or monocytes (Fig. 2C, left and right panels). Similarly, labeling of PRI, GRANTA, or JEKO cell lines with an anti-CD20 conjugated to APC-Cy7 dye revealed that the decoupling rate was different in three B cell lines, without affecting cell viability (Supporting Information Fig. S4).

These results gave evidence for APC-tandem dye degradation as being cell type-dependent.

Degradation of APC-Cy7 and APC-H7 Fluorochromes Is Not Dependent on Light Exposure

Light exposure is a well-known factor influencing the quality of fluorochrome emission. To test whether APC-tandem dyes were sensitive to a photo-induced process, immunolabeled blood cells were either exposed to visible light or incubated in the dark. As shown in Supporting Information Figure S5A, the percentage of degradation of APC-Cy7 and -H7 fluorochromes for the three cell types was similar in the absence or presence of light over a short period of time. This lack of effect of light on degradation could come from a faster bleaching of the APC dye when compared with APC-Cy7 or -H7 dyes. We tested this hypothesis through immunolabeling of beads with anti-CD45 antibodies conjugated to APC-Cy7,

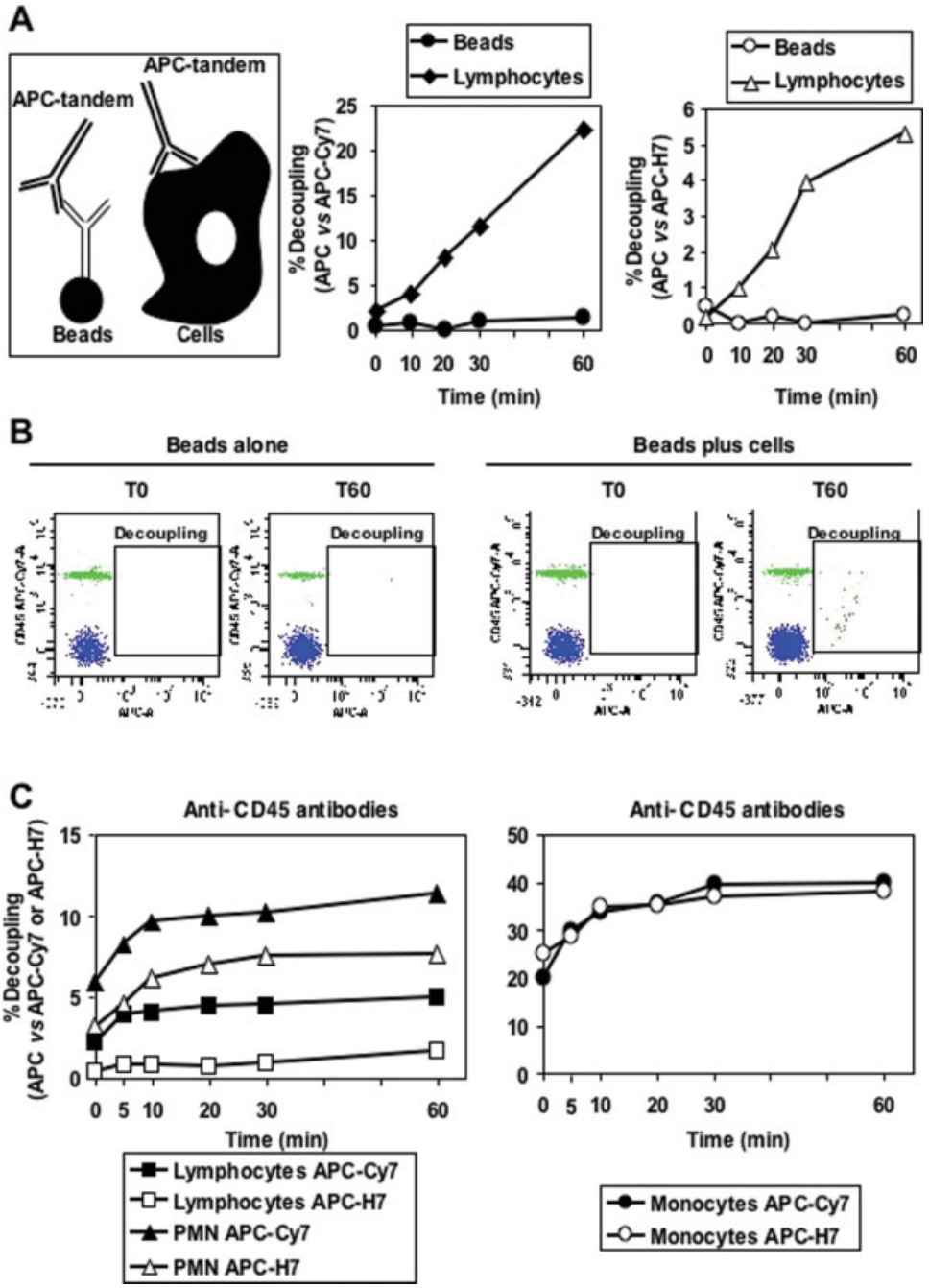

Figure 2. APC-Cy7 and APC-H7 tandem fluorophore degradation is dependent on cell type. (**A**) Left panel presents a scheme of immunolabeled beads and cells (see details in the text). Middle and right graphs display the percentage of decoupling of APC-Cy7 and APC-H7 fluorophores, respectively, on beads and lymphocytes over time. (**B**) Beads were immunolabeled with an anti-CD45 antibody conjugated to APC-Cy7 fluorochrome, washed, and incubated without (left side) or with unlabeled cells (right side). At time 0 (T0) and 60 min (T60), fully compensated dot plots APC vs. CD45-APC-Cy7 show CD45 APC-Cy7-positive (top events) and -negative (bottom events) beads in the APC channel (square called ''decoupling''). (**C**) Blood cells (lymphocytes, PMN, and monocytes) were immunolabeled with anti-CD45 antibodies, percentages of decoupling were determined at various times points and graphed. The left panel shows the degradation of tandem APC-Cy7 in lymphocytes (black squares) and PMN (black triangles) and of tandem APC-H7 in lymphocytes (white squares) and PMN (white triangles). The right panel displays percentage of decoupling in monocytes for tandems APC-Cy7 (black circles) and APC-H7 (white circles). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

APC-H7, or APC. MFIs of the three dyes measured over time decreased gradually with similar kinetics in the absence or presence of a visible light exposure, suggesting that the absence of light effect was not related to an underestimation of the APC fluorochrome bleaching (Supporting Information Fig. S5B). Altogether, these experiments strongly argue against any role

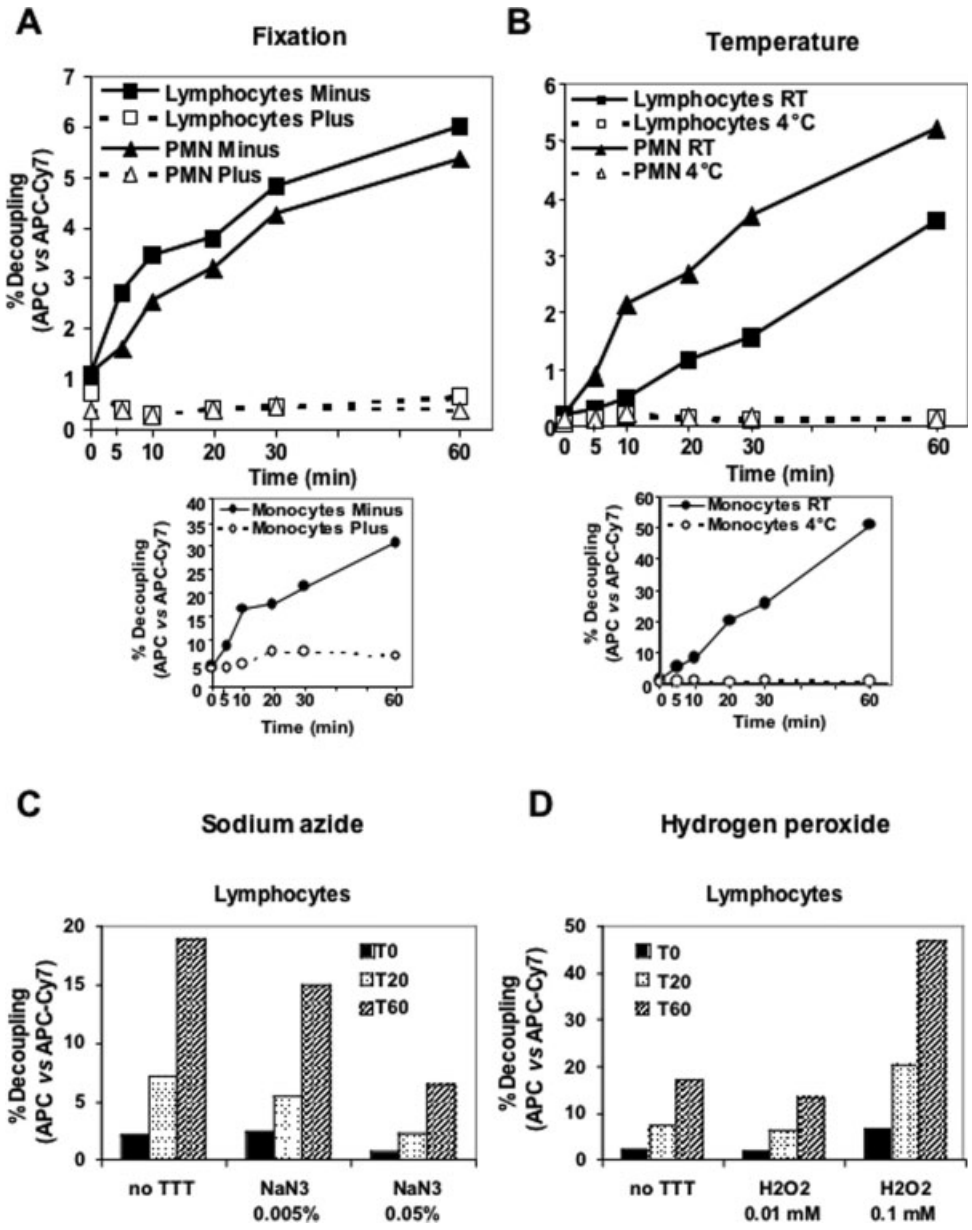

Figure 3. The APC-Cy7 tandem fluorophore degradation is decreased by cell fixation, low temperature, and sodium azide but is increased by hydrogen peroxide. Blood cells were immunolabeled with APC-Cy7-conjugated anti-CD45 antibody, washed, and then incubated at room temperature with a fixative buffer (**A**), at 4°C (**B**), with sodium azide (NaN_3; 0.005 and 0.05%) (**C**), and H_2O_2 (0.01 and 0.1 mM) (**D**). Percentage of decoupling was calculated during the chase and graphed.

of light exposure as a catalyst of APC-tandem degradation in our system.

APC-Cy7 Tandem Fluorophore Degradation Is Decreased by Cell Fixation, Low Temperature, and Sodium Azide But Increased by Hydrogen Peroxide

On the basis of these results, we next investigated possible ways to modulate the cell-dependent degradation of the APC-tandem dyes.

First, cells labeled with anti-CD45 antibodies coupled to APC-tandem dyes were fixed before assessment of fluorochrome degradation over time. As shown in Figure 3A, in such fixation conditions, percentage of decoupling of both APC-tandem dyes remained stable over time and close to the basal value (T0) in lymphocytes and polymorphonuclear leukocytes (top panel, open squares and triangles, respectively) as well as in monocytes (bottom panel, open circles). The strong inhibition of the decoupling phenomenon in the presence of a fixation buffer, which stops metabolic activity, demonstrated the role of cellular metabolism in APC-tandem dye alteration.

Second, we tested in similar leukocyte labeling experiments, the effect of the temperature by performing the incubation at 4°C. Results revealed that at low temperature, all the percentages of degradation of the APC-tandem dyes in

lymphocytes, polymorphonuclear leukocytes (Fig. 3B, top panel), and monocytes (Fig. 3B, bottom panel) remained very low with no increase over time when compared with the percentages observed at room temperature. These experiments confirmed, once again, the importance of cellular metabolism on the APC-tandem fluorochrome degradation.

Then, to block cell metabolic activity when cells were kept at room temperature, we tested the effect of NaN_3. In lymphocytes (Fig. 3C), 0.005% NaN_3 weakly affected the decoupling phenomenon. However, 0.05% NaN_3 strongly inhibited APC-Cy7 alteration. Similar effects were observed in monocytes and PMN, without affecting cell viability (data not shown). This demonstrated that inhibiting metabolic activity led to APC-tandem conjugate protection.

In contrast, we decided to induce APC-tandem dye degradation. Our results (Fig. 2C) revealed that the APC-tandem degradation occurred in higher proportion in monocytes when compared with PMN and lymphocytes, suggesting that the decoupling phenomenon might be dependent on the properties of the cell type studied. One of the major functions of monocytes and macrophages is phagocytosis and killing of microorganisms, which are dependent on the production of superoxide and H_2O_2 (20). To test whether H_2O_2 could potentiate APC-tandem dye degradation, we immunolabeled cells with anti-CD45 antibody conjugated to APC-Cy7 and incubated them with or without H_2O_2 (Fig. 3D). Cells were chased for 20 and 60 min. We observed that 0.01 mM H_2O_2 did not modify the percentage of decoupling in lymphocytes, whereas 0.1 mM H_2O_2 enhanced it. A comparable role of the H_2O_2 was observed in monocytes and PMN, without any effect on cell viability (data not shown), suggesting that the presence of free radicals could play a role in the decoupling phenomenon. Of note, over time, H_2O_2 was able to induce the decoupling of APC-Cy7-conjugated antibodies bound to beads (Supporting Information Fig. S6).

The APC-Cy7 Tandem Fluorophore Protection by Vitamin C in Mono- and Multiparametric Flow Cytometry

On the basis of the multiple drawbacks of using cell fixation, low temperature, and NaN_3 to inhibit APC-tandem dye degradation, we tested the use of vitamin C, known as a free-radical scavenger. In a first series of experiments, we determined the minimal and efficient vitamin C concentration on decoupling APC-tandem conjugate alteration and confirmed that this concentration had no effect on cell viability or on cell membrane expression of the targeted protein (1 mM vitamin C; Supporting Information Fig. S7). In monoparametric experiments, vitamin C strongly inhibited the percentage of decoupling over time in lymphocytes and polymorphonuclear leukocytes (Fig. 4A, left panel) as well as in monocytes (Fig. 4C, right panel). In multiparametric experiments, results of the Figure 4B show that the decoupling phenomenon also occurred on CD3 APC-Cy7-positive T lymphocytes, and this degradation was robustly decreased by incubating the cells with vitamin C (Fig. 4B). The protective effect of vitamin C was confirmed on six different samples (Fig. 4C), without affecting the proportions of peripheral blood cell subsets and the levels of expression of the cell surface markers (Supporting Information Fig. S8A). These data demonstrate that vitamin C might be used to prevent apparition of an abnormal APC signal through degradation of APC-tandem dyes in mono- and multiparametric flow cytometry (Fig. 4D).

Discussion

Conjugation of the near-infrared dyes Cy7 or H7 to APC to form new fluorochromes allows cytometer users to expand their dye-conjugated antibody panels (11–13). However, use of these APC-tandem fluorochromes may suffer from some pitfalls, such as stability and spectral bleed-through of donor dye that may generate false-positive data in the APC channel (15). This emission leads to false-positive events and reduced accuracy of the APC-tandem dye labeling. Until now, emission in the APC channel could be explained by at least two phenomenons. First, a nonoptimal association of the APC and Cy7/H7 dyes may lead to a basal APC fluorescence leakage reflecting an incomplete quenching by Cy7 or H7 fluorochromes. Second, lack of stability of the APC-Cy7/H7 tandem dyes may result from a loss of integrity of APC-Cy7/H7 tandem dyes, which leads to a decrease of the FRET between APC and Cy7/H7 fluorochromes, and a subsequent additional acquired "parasite" APC signal. In our cellular model, the results demonstrated that APC-tandem dye-positive cells gave rise over time to APC-positive cells, whereas APC-tandem dye-positive beads did not lead to APC-positive beads. During the kinetic of decoupling, the APC signal was visualized either in the absence or presence of correct compensations. Indeed, this signal was still detected when compensations were set at higher levels than those obtained with beads (Supporting Information Fig. S9A). This strongly suggested that the APC signal observed was not a spillover of APC-Cy7/H7 dyes but an APC signal itself resulting from a decreased FRET between APC and Cy7 dyes of the APC-tandem conjugates, over time. Additionally, the level of APC-tandem decoupling correlated with the observed intensity of the APC signal, which led to some spillover in the PerCP-Cy5.5 channel; the latter was easily compensated (Supporting Information Fig. S9B). This strengthens our model that the observed APC signal was not due to spillover but a real APC signal.

To overcome this decoupling problem, we closely examined the underlying APC-tandem dye degradation. Based on our study, three lines of evidence stand for a cell metabolism-dependent APC-tandem dye alteration. First, the lack of stability of conjugated antibodies bound to cells (Fig. 1 and Supporting Information Fig. S1), but not to beads (Figs. 2A and 2B, Supporting Information Fig. S6), demonstrates that the parasite signal observed in the APC channel was not dependent on the intrinsic instability of the fluorochromes used in our experimental conditions, but was rather dependent on the presence of cells (Fig. 2B). Second, the percentage of degradation of the APC-tandem dyes was variable among lymphocytes, monocytes, and granulocytes (Fig. 2C), as well as cell lines (Supporting Information Fig. S4), providing evidence for a degradation process that is cell-type dependent.

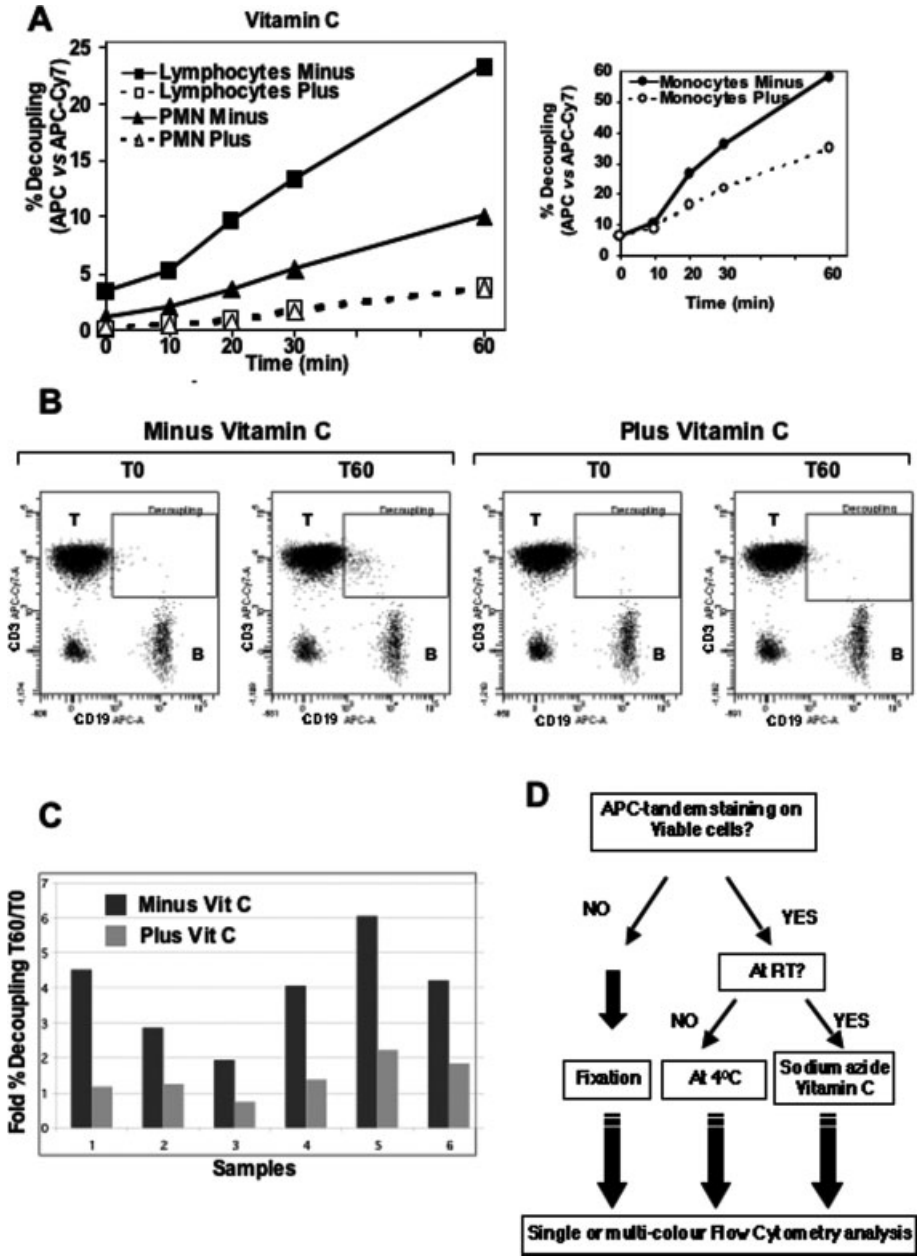

Figure 4. The vitamin C protects cells from APC-Cy7 tandem fluorophore degradation in single and multicolor labeling. Blood cells were immunolabeled with anti-CD45 antibody, washed, and then incubated with 1 mM vitamin C (**A**). Percentage of decoupling was calculated during the incubation and graphed. For better clarity, lymphocytes and PMN are displayed on the same graph (left panel) and monocytes are shown on separated graph (right panel). (**B**) Blood cells were immunolabeled with a combination of antibodies (CD14 conjugated to FITC; -CD20 to PE, -CD5 to PerCPCy5.5, -CD33 to PE-Cy7, -CD19 to APC, and -CD3 to APC-Cy7) (B). Fully compensated dot plots APC vs. CD45 APC-Cy7 show T lymphocytes (T; CD3 APC-Cy7-positive/CD19 APC negative), natural killer cells (NK; CD3 APC-Cy7 negative/CD19 APC negative), and B cells (B; CD3 APC-Cy7 negative/CD19 APC positive) in the APC channel, at time 0 (T0) and 60 min (T60), in the absence (left panels) or presence of 1 mM vitamin C. CD3 APC-Cy7-decoupled cells are shown in white square (called ''decoupling''), which was defined as a threshold at T0 in the untreated cells. (**C**) Ratio of percentage of decoupling at T60 to T0 (fold % decoupling T60/T0) was determined in T lymphocytes from six blood samples in the absence (minus VitC) or presence of 1 mM vitamin C (plus VitC) and graphed. (**D**) The decision tree indicates some guidelines based on our results for flow cytometer users using polychromatic protocols that include antibodies conjugated to APC-tandem dyes.

Third, paraformaldehyde fixation, low-temperature, or NaN_3 (Figs. 3A–3C) incubation prevented the APC-tandem dyes from degradation by, respectively, stopping or strongly reducing the metabolic activity of immunolabeled cells.

At this stage of the study, the molecular mechanism responsible for APC-tandem dye degradation on immunolabeled cells remains to be established. It might involve free radicals because decoupling of APC-tandem conjugates was increased on

cells (Fig. 3D) as well as on beads (Supporting Information Fig. S6) by H_2O_2 addition and was strongly decreased on cells by the addition of a radical scavenger vitamin C (Figs. 4A–4C).

The protective effect of vitamin C on APC-tandem dye conjugated antibodies bound to cell surface markers was also found using multicolor labeling. Therefore, controlling nonspecific signal with vitamin C might increase the accuracy of our assays by decreasing the appearance of APC signal resulting from APC-tandem dye degradation. Thus, vitamin C might be routinely used for flow cytometry evaluation in cellular proteomics of laboratory diagnosis. Interestingly, results of multicolor labeling showed that PE-Cy7 tandem dye conjugated to cell surface marker antibody (anti-CD33) seemed to be subjected to a decoupling phenomenon (Supporting Information Fig. S8B). Indeed, cells labeled with anti-CD33 conjugated to PE-Cy7 tandem fluorochrome led over time to an unexpected PE signal. The latter was higher in monocytes when compared with the one observed in polymorphonuclears (Supporting Information Fig. S8C, left panel). However, addition of vitamin C had a weak and fragile protective effect on the PE signal apparition in monocytes and PMN, respectively (Supporting Information Fig. S8C; comparison of left and right panels). To confirm that PE-Cy7 decoupling is antagonized by vitamin C addition, further studies should be performed with Pan-leukocyte CD45 antibody conjugated to PE-Cy7.

This study allowed us to design guidelines (Fig. 4D) to overcome the issue of tandem instability. Depending on the requirements of the experimental procedure, different possibilities may be applied. When the experiment does not require the use of living cells, a fixation procedure can be used, provided that the antigens of interest are not formalin sensitive. When functional experiments are needed or impaired staining due to fixative-dependant marker-degradation might become relevant, the staining procedure may be performed at 4°C. Alternatively, when the labeling step should be performed at room temperature, addition of the antimetabolic agent NaN_3 or the antioxidant vitamin C is then recommended. Moreover, on the basis of our results, it appears that tandem instability could also impact on the cytometer setting procedure. Indeed, microparticles are accurate reagents for compensating fluorescent spillover (16), but this procedure disregards APC-tandem dyes degradation observed on immunolabeled cells over time. Although compensation is a well-understood procedure in flow cytometry data analysis, it is important to appreciate the intrinsic limits of our data (21,22). On the basis of our data, it is recommended to use single-APC-Cy7/H7 stained cells, as an additional control, to define the precise level of the "parasite signal" in the APC channel inherent in the experimental conditions and to correctly adjust compensation settings.

Therefore, on the basis of this original study, we provide multiple solutions to circumvent the crucial problem of APC-tandem dye alteration depending on the kind of experiment planned.

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